# PRESSURE AND PROTEIN DENATURATION

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Kinetic analyses have indicated that moderate hydrostatic pressures, up to some 700 atmospheres, oppose reversible and irreversible denaturations of certain enzyme systems, apparent at temperatures above the normal optimum of the enzyme reaction, as well as at lower temperatures in the presence of denaturants such as alcohol (1–4). Qualitative observations have shown that such pressures also retard the precipitation of highly purified human serum globulin and egg albumin at 65° (5) and slow the destruction of specific antitoxic activity at the same temperature (6). In this study we have obtained quantitative data with regard to the influence of various pressures, up to 10,000 pounds per sq. in., and of low concentrations of ethyl alcohol on the time course of precipitation of human serum globulin[1] at 65° and pH 6.0.

## EXPERIMENTAL

### *Methods*

Solutions of approximately 2 per cent globulin were made in 0.8 per cent NaCl containing 0.01 M phosphate buffer, pH 6.0. Portions of the stock solution were generally diluted to one-third with the buffered salt solution, and then distributed in 100 × 13 mm. test-tubes. For denaturation at normal pressure, the tubes were stoppered and placed in a water bath at 65° ± 0.02°. Similar tubes were completely filled with portions of the same solution, closed with rubber stoppers, and placed in a water-filled, steel pressure chamber which was then attached to a hydraulic pump. The desired pressure was applied and the entire chamber was placed in the same water bath. Temperature equilibration required $2\frac{1}{2}$ minutes outside, and $5\frac{1}{2}$ minutes inside the pressure chamber. The lag

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[1] The preparation of human serum globulin was obtained through the kindness of Professor E. J. Cohn of the Harvard Medical School. Electrophoretic analysis indicated 94 per cent $\gamma$-, 3 per cent $\alpha$-, and 3 per cent $\beta$-globulin. The preparation contained pseudoglobulin as well as euglobulin. It was made under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

in heating, for which allowance in timing was always made, introduced a source of error because of the changing rates of reaction with rise in temperature. Because of the high temperature coefficient of denaturation, however, this error was small, except with very brief periods of heating. Substantial agreement (within 5 per cent) was obtained between the amount of precipitate in tubes heated for an equivalent period of 30 minutes outside the pressure chamber and inside the chamber under a pressure of only 100 pounds per sq. in. At the end of the designated period of heating, the pressure chamber was placed in a water bath at room temperature, which very quickly cooled the specimens to a temperature which gave rise to no appreciable further denaturation. The tubes that were not subjected to pressure cooled sufficiently rapidly in air on removal from the 65° water bath.

After being heated, the solutions were centrifuged for 20 minutes at 25,000 times gravity, while the rotor was maintained at a low temperature by dry ice placed on the top of the centrifuge. The precipitates were discarded and the supernatants analyzed for the nitrogen in solution by micro-Kjeldahl determinations. In most cases the supernatant showed a distinct opalescence. This was much less noticeable in the specimens containing alcohol. The error caused by the presence of small amounts of denatured protein remaining in suspension was very small, however, except when the total amount of denaturation was slight, as with short periods of heating such as 5 to 10 minutes, or with longer periods of heating under high pressures.

*Influence of Initial Protein Concentration and Products of Denaturation on Time Course of Reaction*

With solutions containing between 2.0 and 0.2 per cent protein at the start, the percentage of the original quantity of protein remaining in solution after 30 minutes at 65° did not vary significantly with the initial concentration. The amount of protein precipitated in this time was usually about 50 per cent of that initially present, with some variation, for reasons not entirely clear, in repeated experiments with different solutions made up from the same lot of the dry globulin. Fig. 1 represents the course of protein denaturation in three solutions prepared as follows: Solution A, from which the other two were prepared, contained 1.85 per cent protein. A portion of Solution A was diluted to a concentration of 0.7 per cent protein, giving Solution B. Solution C was obtained by heating a portion of Solution A for 30 minutes at 65°, centrifuging, discarding the precipitate, and saving the supernatant. The three solutions were stored for 1 week at 3–6°, and then treated at 65° simultaneously. Fig. 1 shows that both the undiluted (Solution A) and diluted (Solution B)

solutions follow the same curve for the percentage of the initial quantity of protein precipitated with time. The partially denatured Solution C, from which the precipitate had been removed, indicates very much the same curve, when the period of preheating and amount of precipitate that was removed earlier are taken into account. The same data for this solution, computed on the basis of the amount of protein in solution at the start of the second heating as 100 per cent, give the uppermost curve, which

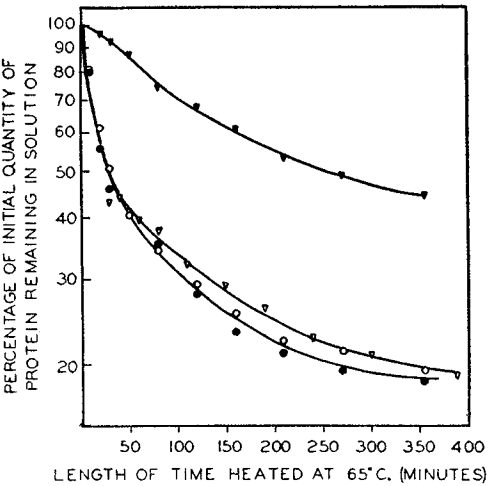


FIG. 1. Denaturation of human serum globulin at 65°. Solid circles, Solution A (see the text) containing globulin in a concentration of 1.85 per cent, before treatment; open circles, Solution B, obtained by diluting Solution A to an initial concentration of 0.7 per cent protein in solution. The triangles refer to Solution C, the supernatant of a portion of Solution A that had been partially denatured by being heated for 30 minutes at 65°. It was then centrifuged, the precipitate was discarded, and the supernatant was stored for 1 week at 3–5°. The solid triangles show the course of precipitation in Solution C with the protein in solution at the start of the second heating taken as 100 per cent. The open triangles illustrate the same data, but take into account the time of denaturation and amount of protein precipitated in the first heating, with the original concentration of 1.85 per cent protein as 100 per cent. The scale of the ordinate is logarithmic.

shows a slight lag and the lack of a relatively rapid denaturation during the first 30 minutes. In other experiments, in which the solutions were first heated for 30 minutes at 65°, then maintained at room temperature for periods of 12 minutes to 4 hours without removal of the precipitate and again heated at 65°, the same curve as that observed for continuously heated solutions was obtained.

The foregoing results indicate that the specific rate of denaturation is first order with respect to the initial amount of protein, and is essentially

independent of the products of the reaction. The shape of the curve, however, shows that the reaction is not unimolecular, but is more complex, as is evidenced by the decreasing rate with time. In these respects the over-all reaction resembles that for the denaturation of diphtheria antitoxin by urea (7) at room temperature, and for the denaturation of anti-*Staphylococcus* hemolysin at 65° under both normal and increased pressure (6). The general shape of the curve is also similar to that for the denaturation of tetanus antitoxin at 65° (8).

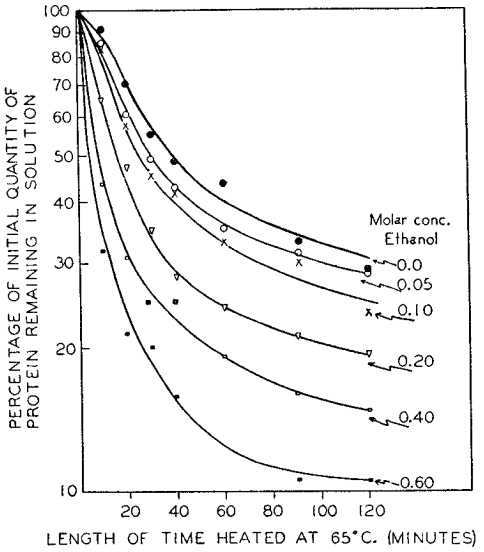


FIG. 2. Influence of alcohol on the rate of precipitation at 65° of globulin solutions having a concentration of 0.69 per cent protein before treatment at 65°. These concentrations of alcohol caused no visible precipitation over long periods of time at room temperature. Logarithmic scale on the ordinate.

*Influence of Alcohol*—Small concentrations of ethyl alcohol increase the rate of precipitation, as is illustrated by the data in Fig. 2 from an experiment with a single stock solution of globulin. The concentration of alcohol required to bring about a precipitation of the protein at room temperature is of the order of 10 times the concentrations that markedly increase denaturation at 65°. The shape of the curve for each concentration is the same, however, if the time scale is changed, as is shown by the straight lines which result when the logarithm of the proportion of the protein originally in solution is plotted against the logarithm of the time of denaturation. These lines have practically the same slope but differ in position on the abscissa (Fig. 3). Thus, in accord with the ob-

servations of Lepeschkin (9) on the similarity of the heat and alcohol denaturation of egg albumin, as well as the recently studied effects of alcohol on the luminescent system (4), the action of alcohol appears to be essentially that of accelerating the denaturation reaction. Preliminary analyses, based on the relation between the logarithm of the concentration of alcohol and the logarithm of the amount of acceleration, indicate that an average of approximately 1.5 more molecules of alcohol are combined with the activated protein molecule undergoing denaturation than with the

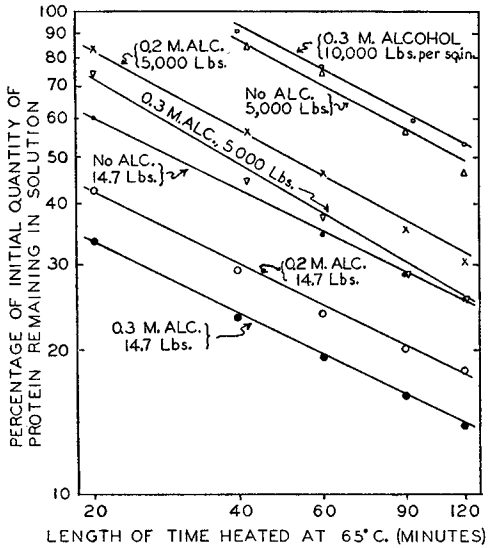


FIG. 3. The data of Figs. 4 to 7, plotted on logarithmic scales as the percentage of the quantity of protein in solution before being heated, against the time of heating at 65°, with and without alcohol, under normal and increased hydrostatic pressure, respectively. Only representative curves are shown to avoid crowding. The scale is logarithmic on both the ordinate and abscissa.

normal molecule. A definite value for this ratio must await clarification of the reason for the apparent change in rate of precipitation during the course of the reaction.

*Action of Hydrostatic Pressure*—Hydrostatic pressure retards the rate of precipitation both in the presence and in the absence of alcohol. The results of a series of experiments with pressures up to 7500 pounds per sq. in. are shown in Figs. 4 to 6, in which the curve for normal pressure in each case represents the average of five repeated determinations. Fig. 7 shows the results of a single experiment with reference to the effects of 10,000 pounds pressure in comparison with atmospheric, and with alcohol concentrations of 0, 0.2, and 0.3 M, respectively. In all cases, the effect of

alcohol is to increase, while that of pressure is to decrease the rate of precipitation. High pressures cause a conspicuous lag in precipitation, which hardly represents a complete initial prevention of denaturation, but rather a very pronounced slowing of a reaction, whereby the concentration of denatured molecules in solution builds up enough to form a precipitate. The source of error referred to earlier no doubt tends to exaggerate this effect; *i.e.*, with very small amounts of precipitate, it is difficult to clarify the solution in the centrifuge. Moreover, a slight evaporation of the solu-

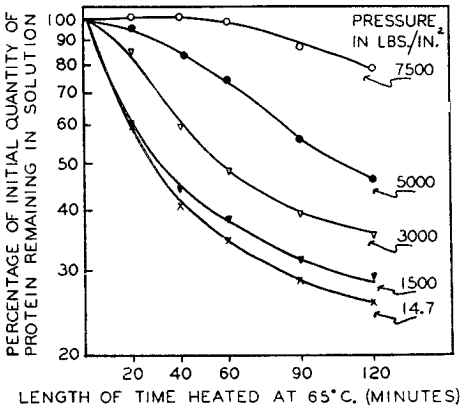


FIG. 4

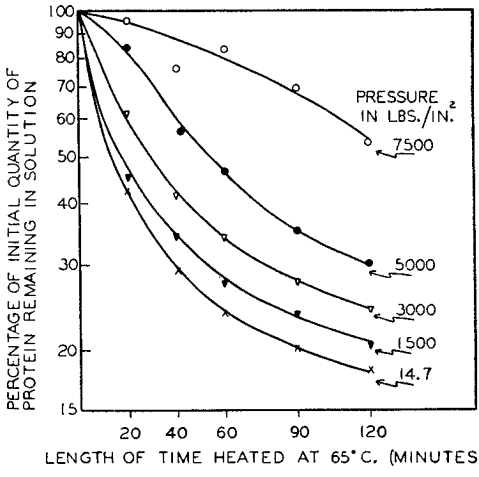


FIG. 5

FIG. 4. The influence of hydrostatic pressure on the rate of precipitation without added alcohol. The curve at normal pressure is the average of five repeated experiments. The data for each of the curves are from a separate pressure experiment. Initial concentration of globulin in the different experiments, from 0.74 to 0.85 per cent. Logarithmic scale on the ordinate.

FIG. 5. The effects of hydrostatic pressure on the rate of precipitation at 65° of globulin solutions containing 0.2 M ethyl alcohol. The curve for normal pressure represents the average of five repeated experiments, the others a single experiment. Logarithmic scale on the ordinate.

tion during the centrifugation would contribute in the same direction to this error. At lower pressures, the rapidity of denaturation makes it difficult to obtain accurate observations during the first few minutes, but when data including the amount of denaturation at the end of the first 10 minutes are plotted with somewhat broader spaces per time unit on the abscissa (*e.g.*, Fig. 2), it is apparent that there is a period of slow precipitation at the start, followed by more rapid precipitation, and a subsequent slowing (*cf.* also Fig. 3).

The changing rates of precipitation with time of heating may be accounted for either by a heterogeneity of the protein molecules, the net

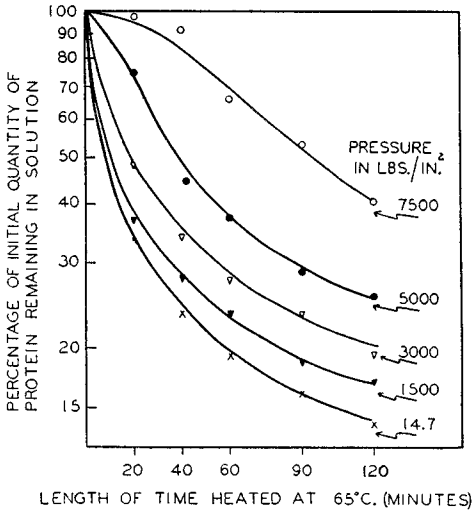


FIG. 6. Influence of hydrostatic pressure on the rate of precipitation at 65° of globulin solutions containing 0.3 M ethyl alcohol. The curve at normal pressure represents the average of five experiments, the others a single experiment. Logarithmic scale on the ordinate.

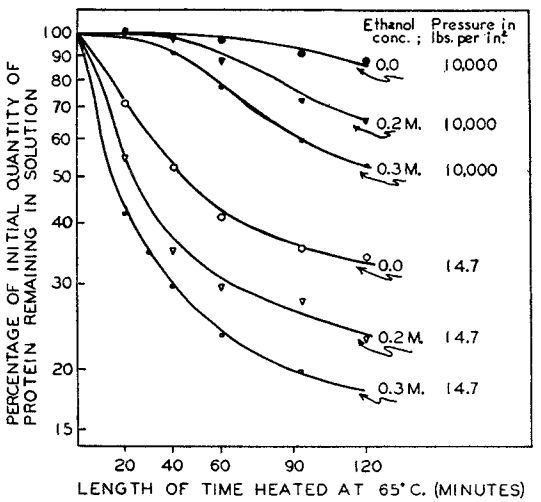


FIG. 7. The rate of precipitation at 65° of globulin solutions, in a concentration of 0.69 per cent protein at the start, containing 0, 0.2, and 0.3 M alcohol, respectively, under normal and 10,000 pounds per sq. in. hydrostatic pressure. The data are from a single experiment. Logarithmic scale on the ordinate.

result representing the summation of reactions with different specific rate constants, or as the result of a complex of reactions with homogeneous molecules, or both. The effects of high pressure in greatly prolonging the

initial time required for the formation of an appreciable amount of precipitate suggest that the first step involves a denaturation reaction with a very large volume change of activation (10). The following experiment provides evidence that a subsequent reaction, possibly an equilibrium, which is characterized by a small volume increase of the final over the initial states, is involved in the precipitation of the denatured protein.

Tubes containing an initial concentration of 0.686 per cent globulin with 0, 0.2, and 0.3 M alcohol, respectively, were treated in triplicate at 65° for 40 minutes. One set was cooled to room temperature, a second set to 4°, in both cases under atmospheric pressure, and the third set to 4° under 10,000 pounds pressure. After 21 hours, the tubes at room temperature were analyzed in the usual manner. The specimens which had been kept at 4° were centrifuged in a rotor precooled approximately to this temperature, and maintained at a low temperature throughout the centrifugation. The results are summarized in Table I, which indicates first that, as might be expected, there is an increase in flocculated protein at low temperatures, and second, that a high pressure opposes this increase. Moreover, the amount of precipitate that has already formed at room temperature may be decreased by subjecting the specimens to high pressure over a long period of time, as is shown by the data in Table II, which are taken from an experiment with a similar series of triplicate tubes. Denaturation was first carried out at atmospheric pressure for 20 minutes at 65° and all tubes were cooled to room temperature. The first set was centrifuged and analyzed very shortly thereafter. The other two sets were maintained for 64 hours at room temperature, one set under normal pressure, the other under 10,000 pounds. From Table II, it is apparent that, within the limits of experimental error, the amount of precipitate did not increase on long standing at room temperature. On the other hand, a significant decrease in the amount of precipitate occurred as a result of high pressure. It is possible, of course, that this effect takes place to some extent through a reversal of the denaturation reaction, but it would seem more likely that it represents an action of pressure on the flocculation of the denatured molecules, in an equilibrium with a negative heat and positive volume change of reaction.

The data in Tables I and II indicate that the pressure effect on the amount of precipitated protein is considerably less marked in the presence of alcohol, in some cases scarcely exceeding the range of experimental error.

With regard to the total reaction, the complexity of the kinetics makes it difficult to arrive at a satisfactory analysis until definitive data are available with respect to the possible significance of heterogeneity of the molecules and the specific reactions that are responsible for the results measured. Heterogeneity is perhaps an important factor in the apparent change in

rate of denaturation during the course of the reaction, thus making it desirable to use as homogeneous a preparation as possible in further studies. Apart from the initial lag, which was conspicuous at high pressures although hardly appreciable at normal pressure, straight lines with a slope of approximately 0.5 result when the logarithm of the percentage of the amount of protein in solution at the start is plotted against the logarithm of the time heated at 65°, with as well as without alcohol, under pressures up to

TABLE I

*Influence of Temperature and Pressure on Amount of Precipitate Formed after Heating 0.686 Per Cent Globulin Solution for 40 Minutes at 65° and Atmospheric Pressure*

| Concentration of ethyl alcohol | Ppt. after standing 21 hrs. at room temperature | | Ppt. after 21 hrs. at 4° | | Ppt. after 21 hrs. at 4° under 10,000 lbs. pressure | | Per cent increase in ppt. at 4° $\left(\frac{B-A}{A}\right)100$ | Per cent decrease in ppt. at 4° under 10,000 lbs. pressure $\left(\frac{B-C}{B}\right)100$ |
|---|---|---|---|---|---|---|---|---|
| | Per cc. protein in solution | Per cent pptd. (*A*) | Per cc. protein in solution | Per cent pptd. (*B*) | Per cc. protein in solution | Per cent pptd. (*C*) | | |
| *M* | *mg.* | | *mg.* | | *mg.* | | | |
| 0.0 | 3.58 | 47.8 | 2.96 | 57.0 | 3.30 | 51.9 | 19.3 | 8.9 |
| 0.2 | 2.42 | 64.8 | 2.09 | 69.6 | 2.27 | 67.0 | 7.4 | 3.7 |
| 0.3 | 2.04 | 70.3 | 1.70 | 75.2 | 1.94 | 71.7 | 7.0 | 4.7 |

TABLE II

*Influence of 10,000 Pounds Pressure for 64 Hours at Room Temperature on Amount of Precipitate from 0.735 Per Cent Globulin Solution Treated 20 Minutes at 65° and Atmospheric Pressure*

| Concentration of alcohol | Per cc. protein in solution | Per cent pptd. (*A*) | After 64 hrs. | | | | Per cent decrease in ppt. under pressure $\left(\frac{B-C}{B}\right)100$ |
|---|---|---|---|---|---|---|---|
| | | | Atmospheric pressure | | Under 10,000 lbs. pressure | | |
| | | | Per cc. protein in solution | Per cent pptd. (*B*) | Per cc. protein in solution | Per cent pptd. (*C*) | |
| *M* | *mg.* | | *mg.* | | *mg.* | | |
| 0.0 | 4.40 | 40.1 | 4.30 | 41.5 | 5.28 | 28.6 | 31.1 |
| 0.2 | 3.12 | 57.5 | 3.10 | 57.8 | 3.55 | 51.7 | 10.5 |
| 0.3 | 2.58 | 64.9 | 2.53 | 65.6 | 2.87 | 61.0 | 7.0 |

5000 pounds per sq. in. Apparently the same relation holds for experiments with pressures of 10,000 pounds per sq. in., although fewer points are available along the curve, since the amount of denaturation was much less. The curves for the over-all reaction are thus largely the same except for the time scale, and the effect of pressure, as judged by these data obtained with the usual procedure, appears to be independent of the alcohol concentration. Furthermore, the magnitude of the pressure effect indicates a large volume increase of activation in the denaturation process,

of the order of 100 cc. per mole of protein, which is of the same order as the value calculated for the luminescent system either in the presence or absence of alcohol (4).

The authors take pleasure in acknowledging the interest as well as lengthy discussions and assistance of Professor Linus Pauling in connection with this study.

## SUMMARY

At 65° and pH 6.0, the denaturation of highly purified human serum globulin at atmospheric pressure takes place at a complex rate which decreases progressively after about 40 per cent of the protein initially in solution has precipitated in the first 20 minutes. To some extent, the change in rate may be due to heterogeneity of the molecules. The specific rate, however, is very nearly the same with initial protein concentrations between 2.0 and 0.2 per cent.

The rate of denaturation is slowed by hydrostatic pressures up to 5000 pounds per sq. in., but the shape of the curve is the same, except for the difference in the time scale. A pressure of 10,000 pounds per sq. in. greatly retards the rate of precipitation and causes a marked initial lag of nearly an hour during which only slight precipitation occurs. The magnitude of the pressure effect indicates a volume increase of activation for the denaturation reaction of the order of 100 cc. per mole of protein in the process of activation of the reacting molecules.

The rate of denaturation at 65° is increased by ethyl alcohol, in relation to its concentration, from 0.05 to 0.60 M, but the shape of the curve remains essentially the same. Pressure retards the denaturation in the presence of alcohol, apparently independently of concentration up to 0.3 M.

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